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TRANSIENT PHOTOCHEMISTRY OF SAFRANIN-O.(U)
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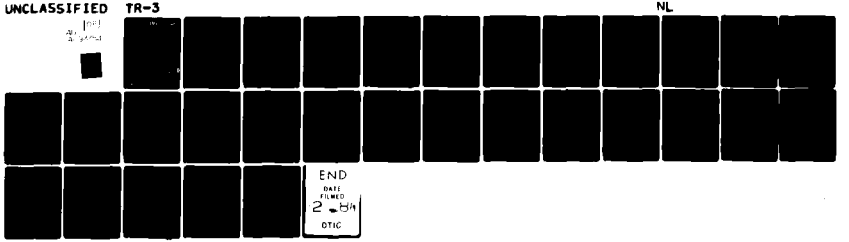

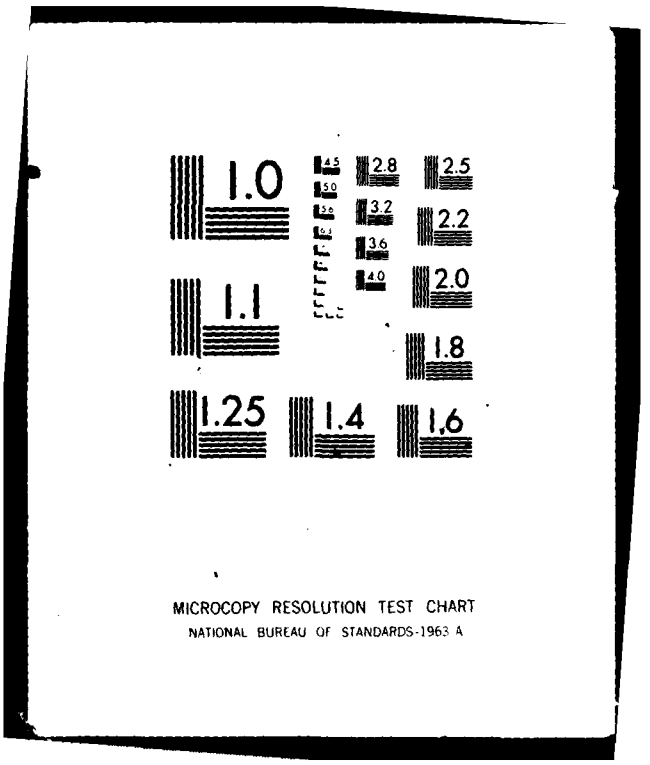
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OFFICE OF NAVAL RESEARCH
Contract No. N00014-78-C-0494
Task No. NR051-679
Technical Report No. 3

Transient Photochemistry of Safranin-O

by

C. E. Baumgartner, H. H. Richtol
and D. A. Aikens

Prepared for Publication
in Photochemistry, Photobiology

Rensselaer Polytechnic Institute
Department of Chemistry
Troy, New York 12181

December, 1980

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: Technical Report #3	2. GOVT ACCESSION NO.: AD-A093 354
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE (and Subtitle): Transient Photochemistry of Safranin-O	5. TYPE OF REPORT & PERIOD COVERED: Interim Technical Report
6. PERFORMING ORG. REPORT NUMBER	
7. AUTHOR(s): C. E. Baumgartner, H. H. Richtol, D. A. Aikens	8. CONTRACT OR GRANT NUMBER(s): N00014-78-C-0494
9. PERFORMING ORGANIZATION NAME AND ADDRESS: Department of Chemistry, Rensselaer Polytechnic Institute, Troy, NY 12181	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: NR051-679
11. CONTROLLING OFFICE NAME AND ADDRESS: Code 472, Office of Naval Research, Arlington, VA 22217	12. REPORT DATE: December 1980 13. NUMBER OF PAGES: 30
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report): unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release: Distribution Unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

Prepared for publication in Photochemistry and Photobiology

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Safranin-O, spectral transients, decay kinetics, pK_as

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

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C. E. Baumgartner, H. H. Richtol*

and D. A. Aikens*

Department of Chemistry
Rensselaer Polytechnic Institute
Troy, N.Y. 12181

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*To whom inquiries should be addressed

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We have characterized the spectra, acidity constants and decay kinetics of the triplet and semireduced radical species of Safranin-O. Between pH 3.0 and pH 10.6 there are three triplet species denoted $^3DH_2^{+2}$, $^3DH^+$ and ^3D, the pK_as being 7.5 and 9.2. All three triplet species exhibit first order decay, the rate constant for $^3DH_2^{+2}$ being circa 5-fold lower than the rate constants of $^3DH^+$ and ^3D. Ascorbic acid and ethylenediaminetetraacetic acid quench the triplet state under appropriate pH conditions and the pH dependencies of the yield of semireduced dye indicate that $^3DH_2^{+2}$ is more reactive than $^3DH^+$ or ^3D. With ethylenediaminetetraacetic acid as reducing agent, there is the additional requirement that at least one of the amino nitrogens be deprotonated to obtain a significant yield of semireduced dye. In these reactions, ascorbic acid is oxidized reversibly, but ethylenediaminetetraacetic acid is oxidized irreversibly, so that with the latter reducing agent photolysis causes buildup of the leucodye, which on subsequent photolysis can reduce triplet state dye. With ascorbic acid as reducing agent, the regeneration of the ground state dye is reversible, the decay of the semireduced radical being second order. In general the transient photochemistry of Safranin-O resembles that of Thionine, the major difference being that the lifetimes of $^3DH_2^{+2}$ and $^3DH^+$ are much longer for Safranin-O than for Thionine.

INTRODUCTION

Although some features of the transient photochemistry of Safranin-O have been established, other aspects critical to full understanding of the photochemistry of this dye remain either known only qualitatively or are unknown. The photogalvanic behavior of Safranin-O with reducing agents was first studied by Kaneko and Yamada (1976, 1977), who found photopotentials in excess of 800 mV with ethylenediaminetetraacetic acid (EDTA) as reducing agent. This value is considerably greater than the 150-250 mV photopotentials observed for the well characterized Thionine-Fe(II)-H^+ system (Sakada et al, 1977; Lichtin et al, 1979), and it is comparable to the ca 800 mV potential characteristic of photosystems I and II in green plant photosynthesis (Hill and Bendall, 1960), but this similarity is more apparent than real. In contrast to the reversible behavior of photosystems I and II, the photochemical oxidation of EDTA is irreversible. With EDTA as the reducing agent, photoreduction of the related dyes Methylene Blue (Merkel and Nickerson, 1954; Oster and Witherspoon, 1957) and Thionine (Bonneau et al, 1973, 1974, 1975) resulted in reversible photobleaching of the dye coupled with irreversible oxidation of EDTA. We have found the photoredox mechanism of the Safranin-O-EDTA system to be similar in many respects.

The mechanism of the Methylene Blue-EDTA photoreaction, which was characterized by Bonneau et al (1973, 1974, 1975) through transient studies, proceeds through the triplet state of the dye. In their proposed mechanism for the Safranin-O-EDTA photoreaction, Kaneko and Yamada (1976, 1977) assumed the same mechanistic path, but they did not examine the transient behavior. Using xenon flash photolysis, Chibisov et al (1967, 1968) reported the absorption spectra of the triplet and semi-reduced species of Safranin-O, and detected five transient species; three triplets and two radicals. They did not report the

acidity constants or the decay kinetics of these transient species, information that is important for proper understanding of the photoreduction process. We have studied in detail the photoreaction of Safranin-O from pH 3.0 to pH 10.6 alone and in the presence of both ascorbic acid and EDTA and have determined the pK values of the triplet and semireduced forms of Safranin-O and the kinetic parameters of their decay.

MATERIALS AND METHODS

Safranin-O (2,8-dimethyl-3,7-diamino-5-phenyl phenazinium chloride) was recrystallized thrice from 50% aqueous ethanol, ascorbic acid was recrystallized from 50% aqueous ethanol and other chemicals were reagent grade materials used without further purification. In all studies, the dye concentration was 2×10^{-6} M in aqueous solution buffered with 1×10^{-3} M phosphate buffer. The dye solution, in a 10 cm quartz or borosilicate cell, was rigorously freed of oxygen by seven successive freeze-pump-thaw cycles under a vacuum of 10^{-5} torr. Spectroscopic transients were secured using the xenon flash photolysis unit described by Strong and Perano (1967), which permitted monitoring the transient absorption between 350 nm and 820 nm beginning 50 μs after flash initiation. Before reaching the sample cell, the flash lamp beam passed through a uranyl nitrate solution and a copper sulfate-Methylene Blue solution to limit the sample illumination to wavelengths between 480 nm and 560 nm, which fall within the wavelength range over which ground state Safranin-O absorbs.

RESULTS AND DISCUSSION

Characteristics of Triplet State

Transient absorption spectra obtained in the absence of reducing agents from pH 3.0 to pH 10.6, representative examples of which are given in Fig. 1,

were used to establish the acid-base properties of the triplet state. Transient spectra cannot be characterized between 500 nm and 600 nm, because the ground state dye absorbs strongly over this wavelength range. At pH 3, the transient spectrum consists of two absorption bands with maxima at 390 nm and 660 nm, both of which diminish in intensity as the pH is raised above 5. Simultaneously, a new band with a maximum above 800 nm appears and grows more intense, reaching maximum intensity near pH 8. With still further increase in pH, the intensity of this new band falls, and a band centered at 420 nm appears and increases in intensity. Except for the transient whose maximum occurs above 800 nm, which was monitored at 800 nm, transients were monitored at the wavelength of maximum absorption, and in the discussion transient spectra are referred to in terms of the wavelength at which they were monitored. No transients are observed over the pH range in the presence of oxygen, and this result together with the lifetimes of the transient species and their reactivity with reducing agents (see below) indicates that they represent triplet species. Because the ground state absorption of Safranin-O is invariant between pH 2.5 and pH 10.6, the three triplet species arise from a common ground state.

Based on the transient decrease in the absorbance of the ground state dye at a wavelength at which the triplet species does not absorb appreciably, the total triplet concentration 50 μs after the flash is 1.1×10^{-6} M at pH 3 and 1.0×10^{-6} M at pH 10. Thus, about 50% of the dye is in the triplet state 50 μs after the flash, a result which differs considerably from the assumption of Chibisov et al (1967) that all of the dye remains in the triplet state several hundred μs following the flash. The concentration of the triplet species was determined from the transient bleaching of the ground state dye, and the molar absorptivity for each triplet species was determined by applying Beer's law to the transient spectrum 50 μs after the flash at a pH that ensures that only one triplet species is present. Following the notation of Bonneau et al

(1973), we have denoted the most highly protonated triplet species, which is the only transient species present below pH 5, as $^3DH_2^{+2}$. In this manner, the molar absorptivity corresponding to the transient absorption at 390 nm is found to be 17,500 $\underline{M}^{-1}cm^{-1}$, and that corresponding to the transient absorption at 660 nm is found to be 21,500 $\underline{M}^{-1}cm^{-1}$. Because the rate constant is the same for the decay of both the 390 nm transient and the 660 nm transient (see Table 1), we attribute both absorption bands to a single triplet species.

The pH dependence of the transient triplet absorption bands indicates that the species $^3DH_2^{+2}$ undergoes stepwise loss of two protons as described by Eq. 1.

$$^3DH_2^{+2} \underset{}{\overset{Ka_1}{\rightleftharpoons}} {}^3DH^+ + H^+ \qquad {}^3DH^+ \underset{}{\overset{Ka_2}{\rightleftharpoons}} {}^3D + H^+ \qquad (1)$$

Molar absorptivities determined for $^3DH^+$ and for ^3D in the same manner as for $^3DH_2^{+2}$ are reported in Table 1. Plots of normalized absorbance vs pH for the species $^3DH_2^{+2}$, $^3DH^+$ and ^3D are given in Fig. 2. At each pH, the absorbance at the monitoring wavelength for each species was divided by the maximum value of the absorbance at that wavelength to normalize the absorbance data for each species. In determining the absorbance of ^3D at intermediate pH values it was necessary to make a minor correction to eliminate a small spectral interference from $^3DH_2^{+2}$. Because the relationship of concentration to normalized absorbance is the same for all three species, the intersections of the curves in Fig. 2 define pK_{a1} and pK_{a2} for Eq. 1, the values being 7.5 and 9.2 respectively. As a check on the correctness of the pK_as, the normalized absorbance plots for $^3DH_2^{+2}$, $^3DH^+$ and ^3D calculated from the pK_as are given in Fig. 2 as solid lines. The close agreement of the theoretical curves and the experimental points confirms the correctness of the pK_a values.

In the absence of quenchers, all three transient spectra exhibit first order decay for approximately three half lives and the first order decay con-

stants for the three triplet species are reported in Table 1. The decay constant of the acidic triplet is noteworthy as it is at least five fold lower than the decay constants of either the intermediate triplet or the basic triplet. As a result of its relatively long life, the acidic triplet is the species most likely to react with ground state dye, and evidence of such a reaction is apparent in the characteristics of the 390 nm transient after ca 1 ms. Whereas the decay of the 800 nm transient at intermediate pH and that of the 420 nm transient at high pH remain first order over the observable lives of the transients, the decay of the 390 nm transient at low pH does not. After approximately 1 ms, the decay becomes second order with a rate constant of $1.5 \times 10^{9} M^{-1} s^{-1}$, which agrees well with that of the acidic form of the semireduced dye (Table 2), and the transient absorption spectrum closely approximates that of this species (Fig. 3). That is, the triplet dye is converted to the semireduced dye, a result we attribute to the reaction of the acidic triplet with ground state dye in Eq. 2. This reaction, which is analogous to the triplet-ground state reactions of thiazine dyes characterized by

$$^{3}DH_2^{+2} + DH^{+} \rightarrow \cdot DH_2^{+} + \cdot DH^{+2} \qquad (2)$$

Kosui et al (1965), yields equimolar quantities of the semireduced dye radical ($\cdot DH_2^{+}$) and the semioxidized dye radical ($\cdot DH^{+2}$).

We have been unable to detect a transient absorption which can be attributed to the semioxidized dye, even when Fe^{+3} or H_2O_2 was present. Both these oxidants rapidly and effectively quench the triplet, but they do not produce a new transient absorption. The failure to detect the semioxidized dye may reflect its lifetime being short, its absorption spectrum falling outside the 300-820 nm range or its rapid reduction by the solvent. In contrast to Chibisov's report (1967), we did not observe the formation of the semire-

duced dye in the absence of reducing agents above pH 7. This may reflect the lower dye concentration in our study which prevents these shorter-lived triplet species from reacting to a significant degree with ground state dye.

The second order nature of the disappearance of the semireduced dye is kinetically consistent with either a disproportionation process yielding leucodye (DH_3^+) and ground state dye (DH^+) such as Eq. 3 or a reaction of the semireduced dye with the semioxidized dye yielding ground state dye directly such as Eq. 5. There is circumstantial evidence which supports both possi-

$$\cdot DH_2^+ + \cdot DH_2^+ \rightarrow DH^+ + DH_3^+ \qquad (3)$$

$$DH_3^+ + 2\cdot DH^{+2} \rightarrow 3DH^+ + 2H^+ \qquad (4)$$

$$\cdot DH_2^+ + \cdot DH^{+2} \rightarrow 2DH^+ + H^+ \qquad (5)$$

bilities, and a clear distinction is not possible. After as many as 50 flashes, the absorption spectrum of the ground state dye remains unchanged, indicating that there is no buildup of the leucodye formed in Eq. 3. If Eq. 3 is the rate controlling step in the disappearance of semireduced dye, the leucodye must be removed by one or more followup reactions which collectively equal Eq. 4. In comparison to this multi-step process, the direct reaction of the semioxidized dye and the semireduced dye described by Eq. 5 is attractive because of its simplicity. On the other hand, the rate constant for the disappearance of the semireduced dye in the absence of reducing agents, $1.5 \times 10^9 M^{-1}s^{-1}$, agrees reasonably well with the value of $1.1 \times 10^9 M^{-1}s^{-1}$ (see Table 2) for the disappearance of the semireduced dye formed in the presence of reducing agents, suggesting that $k_3 \approx k_5$. In the presence of reducing agents it is extremely doubtful that the semioxidized dye exists, so that the rate constant data suggests a mechanism based on Eq. 3.

Semireduced Dye Radical

We have examined the reduction of triplet state Safranin-O to the semi-

reduced dye radical by both ascorbic acid, whose oxidation is reversible, and EDTA, whose oxidation is irreversible. Although the reactions of these two reducing agents with triplet Safranin-O differ in some respects, they are the same in others, and, of course, the intrinsic properties of semireduced Safranin-O do not depend on the nature of the reducing agent. These aspects of the semireduced dye which are independent of the reducing agent are reported below and those which are dependent on the reducing agent are reported in subsequent sections.

Under appropriate pH conditions, a 100-fold excess of ascorbic acid or EDTA reduces triplet Safranin-O to the semireduced dye. Fig. 3 shows representative transient spectra of the semireduced dye obtained at key pH values 1 ms after the flash. Below pH 8.5, the spectrum consists of two absorption bands at 375 nm and 660 nm, whereas above pH 10.5, it consists of only a single band at 430 nm. Both the 375 nm band and the 660 nm band exhibit second order decay with the same rate constant ($1.1 \times 10^9 \underline{M}^{-1}s^{-1}$) and they are therefore assigned to the same species. Thus, there are two radical species connected by a single proton transfer step. Molar absorptivities for the two radical species and the pK_a of the acidic radical species, determined as described for the triplet species, are given in Table 2. The dissociation of the acidic radical can be described by either Eq. 6 or Eq. 7. To distinguish between these two possibilities, we have used the kinetic salt effect under

$$\cdot DH_2^+ \rightleftarrows \cdot DH + H^+ \qquad (6)$$

$$\cdot DH \rightleftarrows D^- + H^+ \qquad (7)$$

conditions which ensure that the acid dissociation equilibrium lies far to the left to determine the charge of the acidic semireduced radical. Because the decay of the semireduced radical is second order with respect to the semireduced radical, a plot of the logarithm of the rate constant <u>vs</u> the square

root of ionic strength should be linear with a slope of 1.02 if the formula of the radical is $^{\cdot}DH_2^+$ and a slope of zero if the formula of the radical is $^{\cdot}DH$. Over the range of ionic strength from 2×10^{-3}M to 0.5M (KNO_3) the experimental plot is linear, with a slope of 0.78, which agrees reasonably well with the theoretical value of 1.02 for the semireduced radical $^{\cdot}DH_2^+$.

Generation of Semireduced Dye by Ascorbic Acid

The extent to which triplet Safranin-O is reduced to the semireduced dye by ascorbic acid is extremely sensitive to the degree of protonation of the triplet species. As estimated by extrapolation of linear second order kinetic plots to zero time, the pH dependence of the concentration of semireduced Safranin-O formed initially by a 100 fold excess of ascorbic acid is shown by the closed circles in Fig. 4. Below pH 5, the conversion of the triplet species to semireduced Safranin-O is essentially complete, but with increasing pH, the yield of semireduced dye drops sharply until at pH 10, it corresponds to only approximately 20% of the triplet species. This pH dependence cannot be attributed to the effect of pH on the distribution of the ascorbic acid species. The pK_as of ascorbic acid are 4.2 and 11.6 (Sommer, 1963) and it is clear that the pH dependence of the concentration of semireduced dye does not correlate with these pK_as. On the other hand, the decrease of the initial concentration of semireduced dye above pH 5 reflects the dropoff of the concentration of the acidic triplet species, $^3DH_2^{+2}$. Because the pK_as of $^3DH_2^{+2}$ and of $^3DH^+$ differ by less than two units and the data scatter somewhat, we have not attempted to analyze rigorously the pH dependence of the yield of semireduced dye. It is striking, however, that the yield of semireduced dye drops by nearly 50% at pH 7.5, which corresponds to the pK_a of $^3DH_2^{+2}$. Because the yield of semireduced dye at pH 7.5 is nearly 50% less than the yield from $^3DH_2^{+2}$, the reactivity of $^3DH^+$ must be much less than that

of $^3DH_2^{+2}$. It is also apparent that the yield of semireduced dye derived from the basic triplet, ^3D, is approximately 15-20% of the yield derived from the acidic triplet $^3DH_2^{+2}$. Thus the reactivity of the Safranin-O triplet species toward ascorbic acid falls in the order $^3DH_2^{+2} > {}^3DH^+ \sim {}^3D$. We attribute the high yield of semireduced dye derived from the acidic triplet species in part to its long lifetime, which the rate constants in Table 1 show to be ca 5 fold longer than the lifetimes of the two more basic triplet species. First order decay of the triplet species to ground state Safranin-O competes with its reduction by ascorbic acid, so that the slower the first order decay of the triplet state, the greater the opportunity for reduction by ascorbic acid. A second factor contributing to the high yield of the semireduced dye at low pH is the significantly higher reaction rate between ascorbic acid and the triplet state of Safranin-O at low pH. At pH 10, the triplet state is clearly detectable 50 μs after the flash, but at pH 4, only the semireduced dye is present at this point. Recently, Vogelmann et al (1976) found that the acidic triplet species of both thionine and lumiflavin undergo reduction more rapidly than the basic triplet species, a result they were able to explain in terms of the more positive reduction potentials of the acidic triplet species. The same phenomenon may be important for Safranin-O.

The probable general nature of the reaction steps which govern the formation and the decay of semireduced Safranin-O when ascorbic acid is the reducing agent is summarized schematically by Eqs. 8-13.

$$DH^+(S_o) \xrightarrow{h\nu} {}^*DH^+(S_1) \tag{8}$$

$${}^*DH^+(S_1) \rightarrow {}^3DH^+(T_1) \underset{-H^+}{\overset{+H^+}{\rightleftarrows}} {}^3DH_2^{+2}(T_1) \tag{9}$$

$${}^3DH_2^{+2}(T_1) + Red \rightarrow {}^{\cdot}DH_2^+ + Ox \tag{10}$$

$$\cdot DH_2^+ + Ox \rightarrow DH^+(S_o) + Red + H^+ \quad (11)$$

$$\cdot DH_2^+ + \cdot DH_2^+ \rightarrow DH^+(S_o) + DH_3^+ \quad (12)$$

$$DH_3^+ + Ox \rightarrow \cdot DH_2^+ + Red + H^+ \quad (13)$$

In these equations $DH^+(S_o)$ and DH_3^+ indicate the ground state dye and leucodye respectively, $^3DH^+(T_1)$ designates any of the three forms of the triplet dye, $\cdot DH_2^+$ represents either of the two forms of the semireduced dye, and Ox and Red denote respectively the oxidized and reduced forms of ascorbic acid.

That the photoreduction proceeds through the triplet state (Equation 10) rather than through the singlet excited state is shown by the fact that addition of the reducing agent does not lower the fluorescence yield, which means that the reducing agent does not lower the population of the singlet excited state. This result confirms the assumption of Kaneko and Yamada (1976, 1977) that, by analogy with thiazine dyes (Bonneau et al, 1973, 1974, 1975), the photoreduction of Safranin-O proceeds through the triplet state. Regeneration of ground state Safranin-O and ascorbic acid is attributed to one or more of the processes defined by Eq. 11 through 13. The decay of the semireduced transient absorption remains second order for at least several milliseconds over the pH range studied, a result consistent with several possible pathways involving these reactions. The rate constant for decay of the semireduced dye is insensitive to the reducing agent concentration over the range 2×10^{-5} M to 2×10^{-3} M, which shows that the further reduction of the semireduced dye to the leucodye by ascorbic acid is not significant. Simultaneous monitoring of the rate of disappearance of the semireduced dye at 640 nm and of the rate of reappearance of the ground state dye at 500 nm indicates that the rate of reappearance of the ground state dye is somewhat slower than the rate of disappearance of the semireduced dye. This result suggests that more

complex pathways than Eq. 11 are important. Despite this difference in rates, however, the absorption spectrum of the ground state dye remains unchanged after 50 flashes, indicating that the regeneration of the ground state dye is complete.

Generation of Semireduced Dye by EDTA

In contrast to the oxidized form of ascorbic acid, which is reversibly reduced, the oxidized form of EDTA does not reoxidize the semireduced radical or the leucodye, so that each flash increases the concentration of leucodye until the Safranin-O is completely bleached. Initially, flash illumination of Safranin-O-EDTA lowers the ground state dye concentration and raises the leucodye concentration to an equal extent. Once a significant fraction of the ground state dye has been converted to the leucodye, however, the accumulated leucodye reduces the triplet Safranin-O formed by subsequent flash illumination, as shown by Eq. 14.

$$^3DH^+(T_1) + DH_3^+ \rightarrow 2{\cdot}DH_2^+ \qquad (14)$$

As a result of the reaction of leuco Safranin-O with triplet Safranin-O, it is difficult to separate the contributions of EDTA and of leucodye to the photoproduction of the semireduced dye. Nonetheless, the ability of leuco Safranin-O to compete with EDTA as a reducing agent even though the concentration of EDTA is more than 2 orders of magnitude higher than that of the leucodye indicates that the reactivity of leucodye toward triplet Safranin-O exceeds that of EDTA.

The effect of pH on the initial concentration of semireduced dye generated with EDTA as reducing agent is shown by the open circles in Fig. 4. To prevent photoreduction of the dye except by flash illumination, the solutions were outgassed in the dark and the analyzing beam was filtered to eliminate absorption by the ground state dye, and to avoid interference from accumulated

leucodye, each sample was subjected to flash illumination only once. The concentration of free radical initially formed was estimated by extrapolation of the linear second order kinetic plots to zero time. Above pH 7, the initial concentration of semireduced dye with EDTA as reducing agent corresponds closely to the concentration obtained with ascorbic acid as reducing agent. As with ascorbic acid, we attribute the decline of the initial concentration of semireduced dye with increasing pH to the lower reactivity of the basic triplet species with the reducing agent. The fall in the initial concentration of semireduced dye with decreasing pH below pH 7 is attributed to the distribution of the EDTA species in this pH range. The pK_a values for EDTA (H_4Y) are 2.1, 2.7, 6.2 and 10.3, (Schwarzenbach and Ackermann, 1947) and because the nitrogens are the most basic sites (Chapman et al, 1962), it is only in the species HY^{-3} and Y^{-4} that one or more electron pairs on the nitrogen are free. Above pH 6.2, HY^{-3} is the predominant species, but at lower pH, HY^{-3} is converted to H_2Y^{-2}, in which there are no free electrons on the amino nitrogens. We therefore attribute the low reactivity of EDTA with triplet Safranin-O below pH 6 primarily to the low reducing power of diprotonated EDTA.

Comparison with Thionine

As might be expected, the transient photochemistry of Safranin-O resembles in many respects that of the related dye Thionine, but there are significant differences. Like Safranin-O, Thionine exhibits 3 triplet species and two semireduced species, and the pK_as are similar to those of Safranin-O (Faure et al, 1967; Bonneau et al, 1968, 1975). Also, the order of reactivity of the Thionine triplet species toward EDTA, which is $^3DH_2^{+2} > {}^3DH^+ > {}^3D$ (Bonneau et al, 1975), is qualitatively similar to the order of reactivity of the Safranin triplet species, which is $^3DH_2^{+2} > {}^3DH^+ \sim {}^3D$. The most striking difference

between Thionine and Safranin-O is the much longer lifetimes of the triplet species of Safranin-O, especially that of the acidic triplet $^3DH_2^{+2}$. Whereas for Thionine, the lifetimes of the triplet species $^3DH_2^{+2}$ and $^3DH^+$ are 8.5 μs and 12 μs (Bonneau et al, 1974), the corresponding values for Safranin-O are 277 μs and 44 μs.

ACKNOWLEDGEMENT

This work was supported in part by the Office of Naval Research.

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FIGURE CAPTIONS

Figure 1 Transient Absorption Spectra of Triplet Species

- ● Acidic triplet $^3DH_2^{+2}$ at pH 3.0
- ● Intermediate triplet $^3DH^+$ at pH 8.3
- ○ Basic triplet ^3D at pH 10.4

Figure 2 Normalized Absorbance - pH Plot for Triplet Species

- ● Acidic triplet 660 nm
- ● Intermediate triplet at 800 nm
- ○ Basic triplet at 420 nm

Figure 3 Transient Absorption Spectra of Semireduced Radicals

- ● Acidic radical $\cdot DH_2^+$ at pH 4.2
- ○ Basic radical $\cdot DH$ at pH 10.65

Figure 4 Effect of Reducing Agent and pH on Yield of Semireduced Radical

- ● Ascorbic acid
- ○ Ethylenediaminetetraacetic acid

Table 1

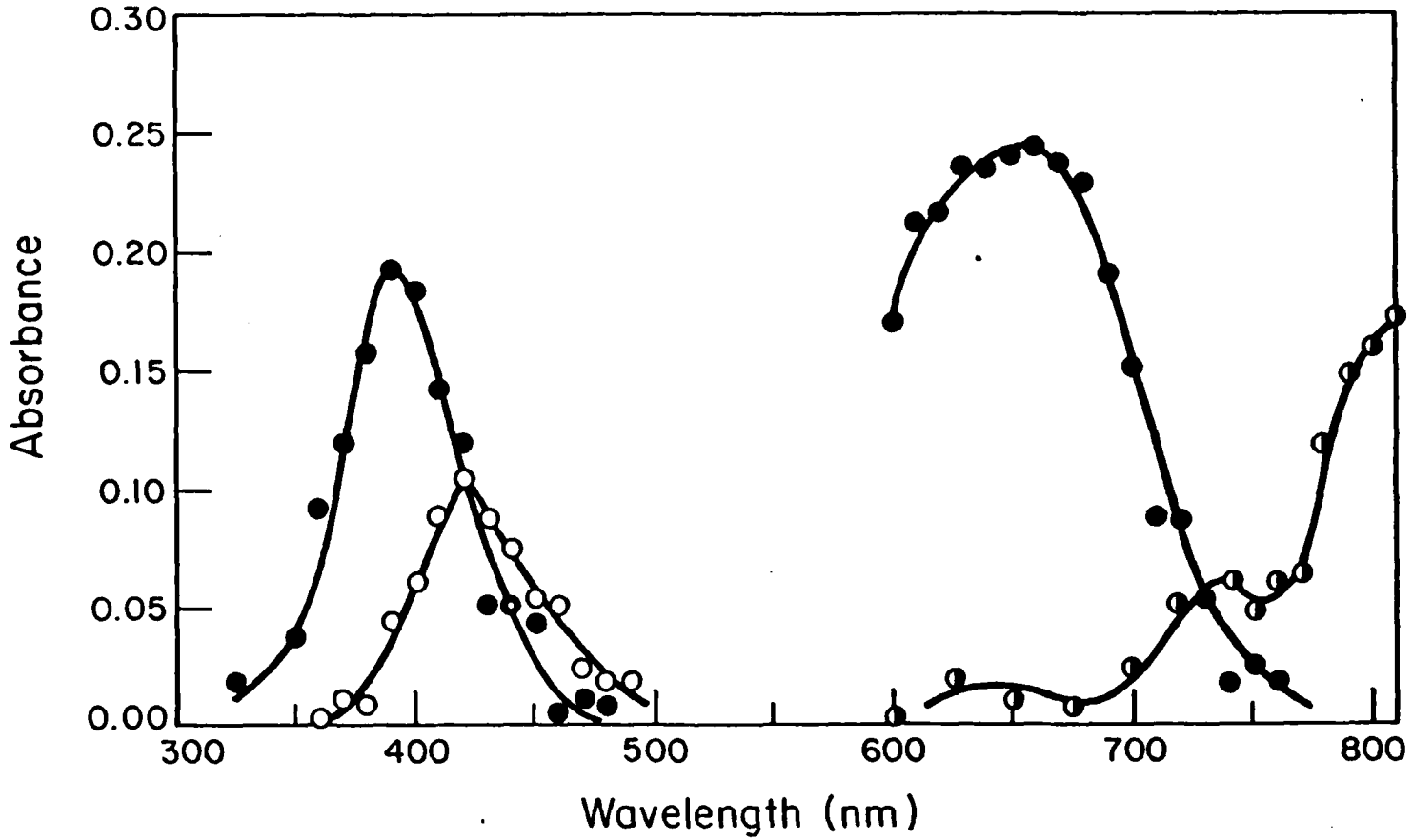
Properties of Safranin-O Triplet Species

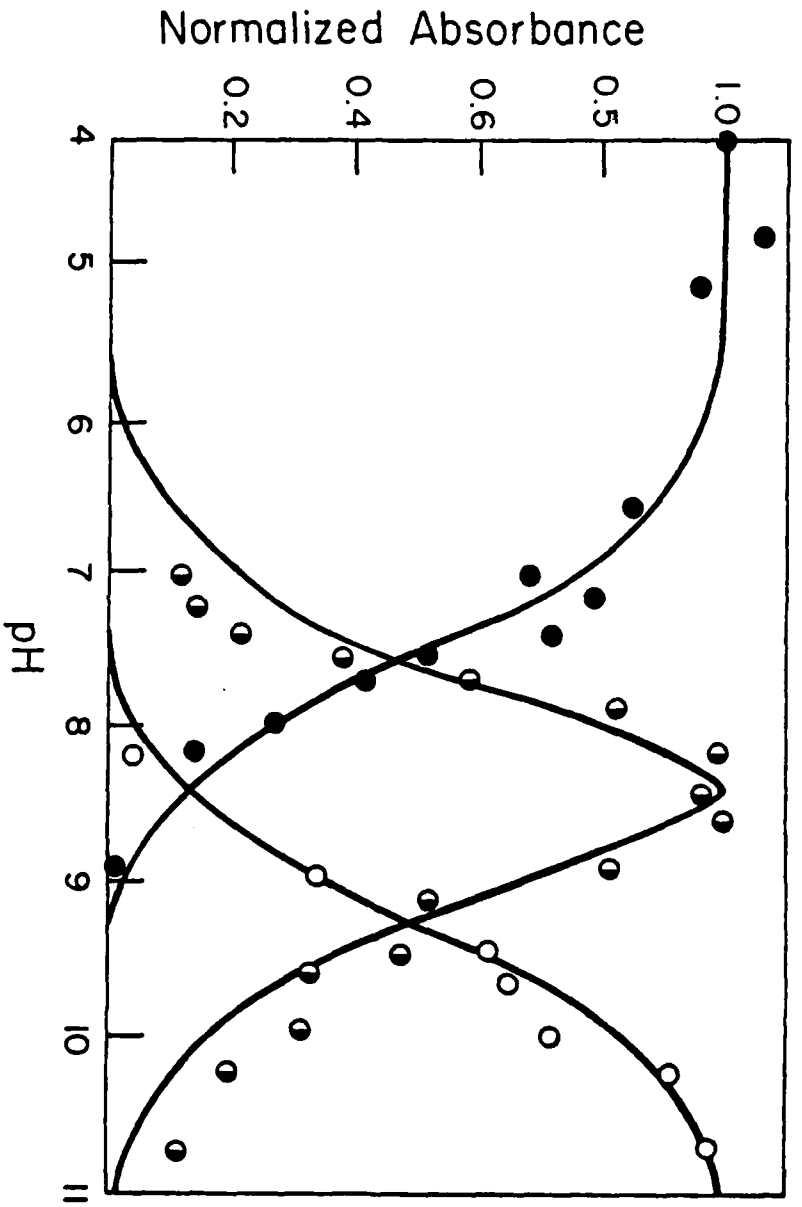
Species	pK_a	Absorption Maximum (nm)	Molar Absorptivity (M^{-1} cm^{-1}	Decay Constant (sec^{-1})
$^3DH_2^{+2}$		390 660	17,500 21,500	3.6×10^3
	7.5			
$^3DH^+$		Above 800	15,500 at 800 nm	2.25×10^4
	9.2			
^3D		420	10,000	1.7×10^4

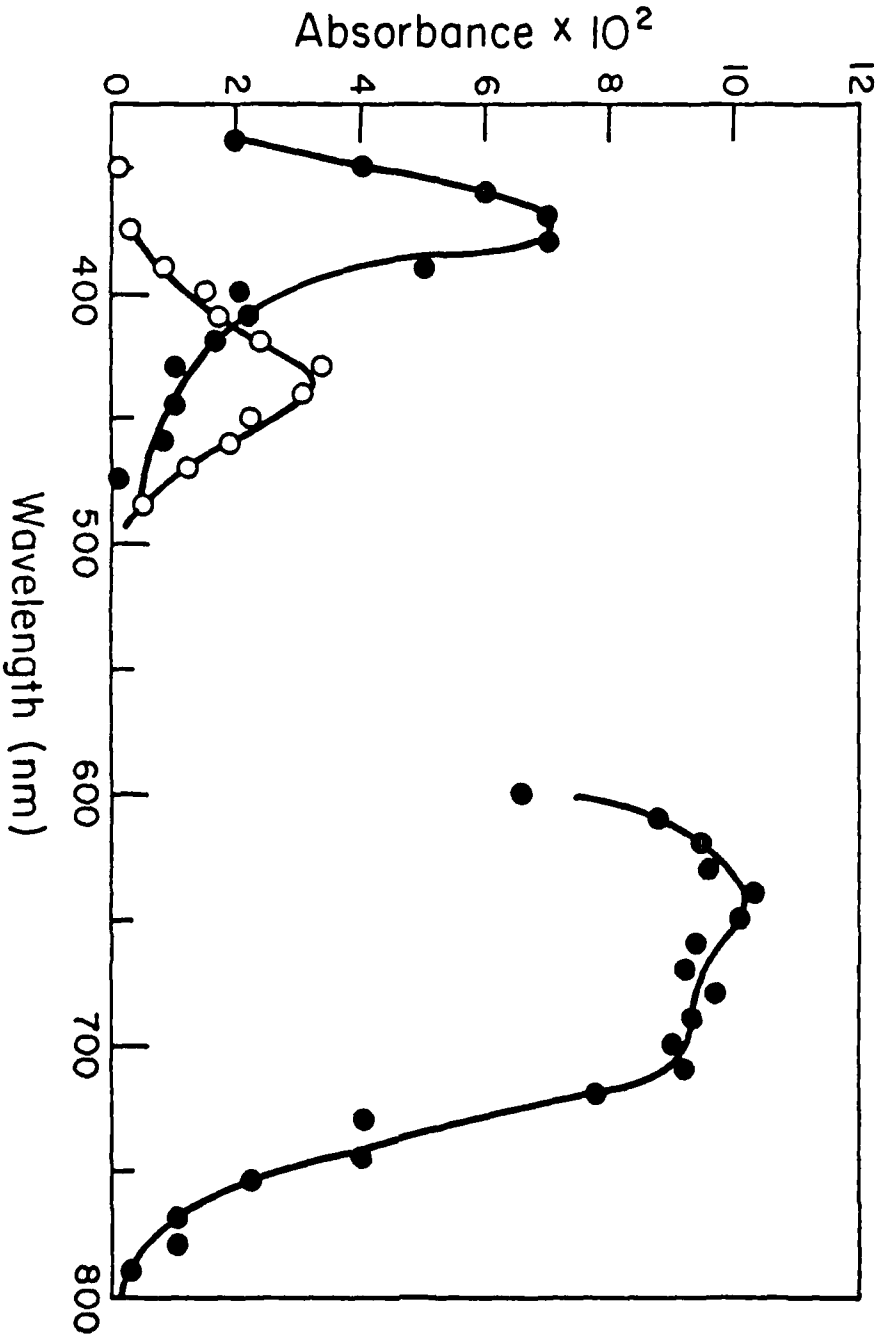
Table 2

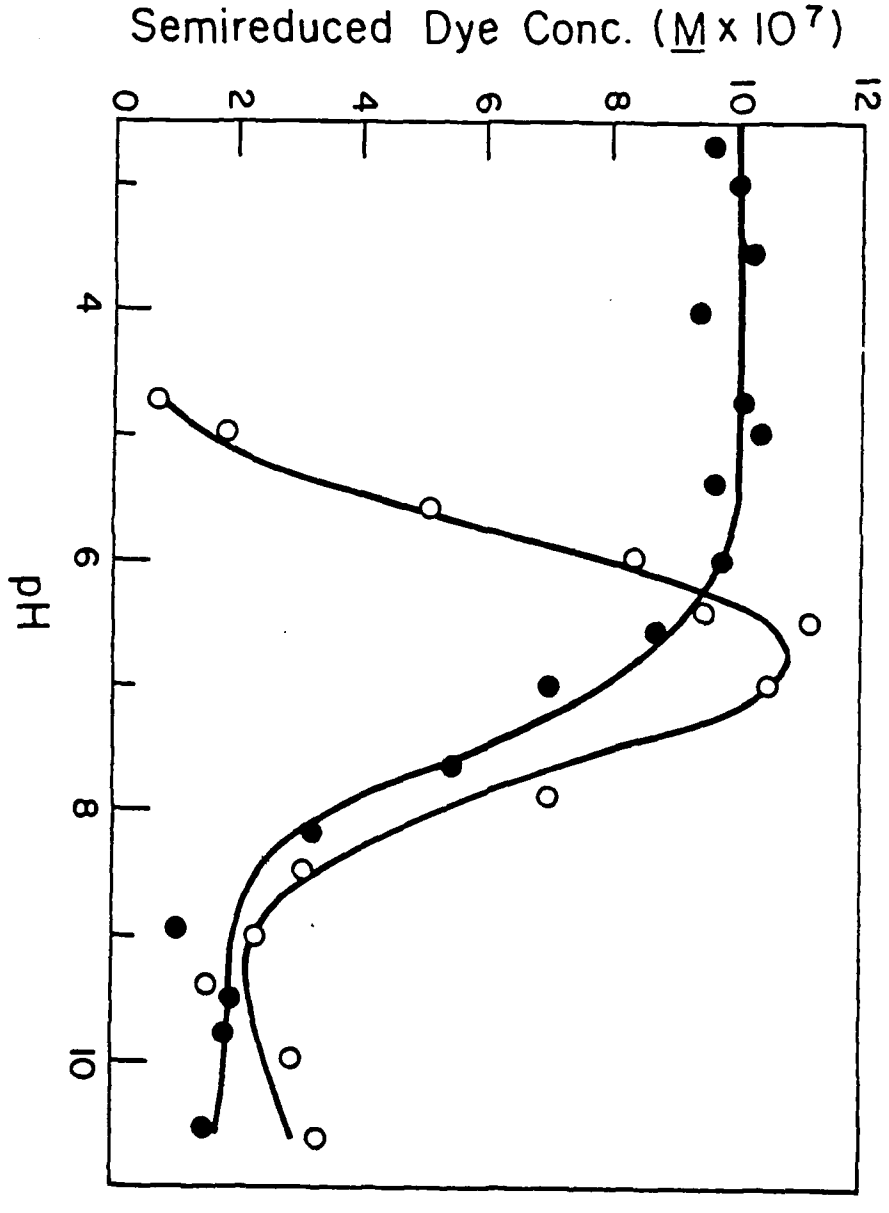
Properties of Semireduced Safranin-O Species

Species	pK_a	Absorption Maximum (nm)	Molar Absorptivity (M^{-1} cm^{-1})	Decay Constant (M^{-1} sec^{-1})
$^{\cdot}DH_2^+$		375 640	7,000 10,000	1.1×10^9
	9.5			
$^{\cdot}DH$		430	13,500	2.5×10^9


0.30
0.25
0.20
0.15
0.10
0.05
0.00
Absorbance
300
400
500
600
700
800
Wavelength (nm)


Normalized Absorbance
0.2
0.4
0.6
0.5
1.0
4
5
6
7
8
9
10
11
pH


Absorbance x 10^2
0
2
4
6
8
10
12
Wavelength (nm)
400
500
600
700
800


Semireduced Dye Conc. (M x 10^7)
0
2
4
6
8
10
12
pH
4
6
8
10

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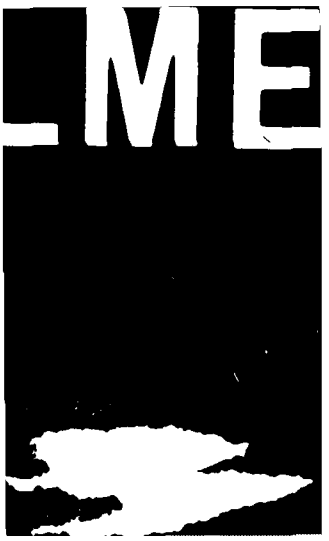
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